THERMOLABILITY OF RAT LIVER NAD-DIAPHORASE ISOENZYMES

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After electrophoretic fractionation of rat liver extract in polyacrylamide gel two isoenzymes of NAD-diaphorase were discovered, one of which was appreciably inactivated by heating to 55° for 10 min.

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The NAD (DPN)-diaphorases are known to play an important role in tissue respiration by supplying the cells with oxidized forms of coenzyme, essential electron carriers from many metabolites.

The results of investigation of this enzyme by histochemical methods have shown that it is mainly localized in the mitochondria, although extramitochondrial diaphorases are sometimes found [5]. This finding suggests the possible heterogeneity of the diaphorases. The existence of isoenzymes, enzymes acting on the same substrate but differing in a number of physicochemical properties, has been demonstrated in the case of lactate dehydrogenase, arginase, acid and alkaline phosphatase, esterases, tyrosine – transaminase, and many other enzymes [1, 4].

The principal method used to study isoenzymes is electrophoretic separation on agar and starch gels. No information concerning the study of isoenzymes of NAD-diaphorase could be found in the literature.

In the present investigation the existence of isoenzymes of NAD-diaphorase in the liver was demonstrated, and their conditions of separation and sensitivity to heat were studied.

EXPERIMENTAL METHOD

In preliminary experiments the optimal conditions of preparation of the liver extracts, of their separation by electrophoresis in polyacrylamide gel, and of performance of the color test in those parts of the gel containing the enzyme after electrophoresis were established.

For the experiments to study the thermolability of the isoenzymes, five noninbred albino male rats weighing about 200 g and receiving the ordinary laboratory diet were used. The animals were killed by decapitation, and the liver was extracted, washed with cold physiological saline, and dried on filter paper. To prepare the extract 1 g liver was homogenized with Tris-glycine buffer, pH 8.3 (0.005 M Tris buffer and 0.037 M glycine), and centrifuged in the cold at 18,000 rpm. The supernatant was diluted with the same buffer to give a protein concentration of 2 mg/ml, after which one part of the extract was left in the cold while the other was heated (with stirring) in a bath at 55° for 10 min. Next, equal volumes of sucrose were added to both parts of the extract, and 0.1 ml of the mixture (0.1 mg protein) was used for fractionation.

Electrophoresis in polyacrylamide gel was performed as described by Davis [3] for 3 h in 8 columns measuring 70 × 6 mm, with an initial voltage of 300 V and current 50 mA, both chambers being cooled. When fractionation was complete, cylinders were cut out of the tubes, and to reveal areas of diaphorase activity they were placed in a mixture prepared in accordance with Burstone's formula [12] as used in histochemistry, with slight modification. The mixture contained Tris-buffer (0.05 M, pH 7.5; 10 ml), NAD · H_2 (0.023 M; 1 ml), and nitro BT (0.009 M; 1 mg).

The samples were incubated in a water bath at 37° for 2 h. Control samples were incubated in a mixture without NAD · H_2. The stained gel cylinders were kept in a mixture of methanol – acetic acid – water (10 : 1 : 30).

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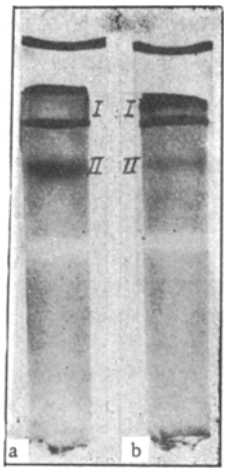

Fig. 1. Electrophoresis of rat liver extract. a) Native extract; b) heated before fractionation for 10 min at 55°.

EXPERIMENTAL RESULTS

After electrophoresis of the rat liver extracts as described above, two zones possessing NAD-diaphorase activity were discovered (see Fig. 1). The first isoenzyme from the starting line (I) has the electrophoretic appearance of a narrow, clearly defined disc and its R_f value is 0.04. The second isoenzyme (II) occupies a wider area with a lower concentration of formazan and has an R_f value of 0.13. In experiments on many rats we never found appreciable individual differences in the relative proportions of these two isoenzymes. Heating the extracts for 10 min at 55° before electrophoresis led to essential changes in electrophoretic behavior of the enzymes: The activity of isoenzyme II was sharply reduced whereas the intensity of the color at the site of isoenzyme I remained as before (see Fig. 1). Heating the extracts for 10 min at 45° caused a slight decrease in the intensity of the color in the region of isoenzyme II, but likewise had no effect on isoenzyme I. Heating for the same period at 65° led to an appreciable reduction is isoenzyme I and to loss of isoenzyme II. Heating the extracts to 90° led to the complete disappearance of both isoenzymes.

These experiments showed that the isoenzymes of NAD-diaphorase not only differ in electrophoretic mobility, but also differ in their resistance to heating. It may be postulated that the synthesis of these proteins is controlled by different genes, or that they are products of different combinations of polypeptide chain subunits, as has been demonstrated for lactate dehydrogenase. According to one report [6], the isoenyzmes of lactate dehydrogenase also possess individual thermolability.

We did not study variations in the physiological functions of each NAD-diaphorase in the cell.

The wide distribution of NAD-diaphorase in all tissues of the body and the changes observed in its activity in pathological states and during experimental procedures [7] suggest that a comprehensive investigations of its isoenzymes would be profitable.

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